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CONTINUOUS-FLOW TOXICITY BIOASSAY OF OIL REFINERY EFFLUENTS

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Continuous-flow Toxicity Bioassay of Oil Refinery Effluents

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SUMMARY STATEMENT

The routine or short-term bioassay is commonly used for evaluating acute toxicity of industrial wastes to fish. The method does not measure chronic or cumulative toxicity resulting from exposure to comparatively low concentrations over a long period. Knowledge of chronic toxication is necessary in determining the concentration of a pollutant which will be safe for aquatic life. The present study was made to determine the effect of oil refinery effluents on fish when applied for extended periods under conditions of continuous renewal. Results from long-term bioassays were compared with those obtained from routine bioassays. Chemical analyses were made on effluents tested and factors affecting toxicity were considered.

Routine and long-term bioassays were made of effluents from four refineries. Routine tests (96 hours) were conducted as outlined in the American Public Health Association Standard Methods (1960). A constant-flow apparatus, capable of renewing test solution every 5.5 hours, was constructed for long-term tests which were 16 or 32 days long. Effluents from two refineries were of low initial toxicity. Chemical characteristics of effluents and test solutions were measured. Changes in toxicity of stored effluents were determined.

Fish were not adversely affected by extended exposure to sub-acute concentrations of effluents with initial high toxicity. High-toxicity effluents did not change appreciably during storage. Low-toxicity effluents decreased in toxicity during storage. Prolonged exposure to sub-acute

concentrations of low-toxicity effluents resulted in appreciably less fish survival than in short-term bioassays. Condition factors of fish surviving extended exposure in sub-acute concentrations of low-toxicity effluents were significantly less than in control fish. Dilution required to prevent chronic toxication appeared to be at least 60 percent. Effluent components responsible for chronic toxication are not known but the effect of potential toxicants is discussed.

A study of the effect of storage on toxicity of oil refinery effluents was published in the October 1961 issue of the Journal Water Pollution Control Federation Vol. 33, No. 10, pp. 1107-1111. Forty copies of this paper have previously been submitted.

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INTRODUCTION

A short-term or routine toxicity bioassay outlined in the American Public Health Association Standard Methods (1960) is coming into general use for evaluating acute toxicity of industrial wastes and other substances to fish. The test is made by preparing serial dilutions of the substance in water and recording survival after 24, 48 and 96 hours of exposure. The method is useful in providing a rapid and economical evaluation of certain wastes. However, routine tests do not measure the effects of continuous exposure to comparatively low concentrations of toxic substances over a long period. Chronic or cumulative toxicity can adversely affect the growth and reproduction of fish or increase their susceptibility to disease.

The present study was made to determine the effects of oil refinery effluents on fish when applied for extended periods under conditions of continuous renewal. Toxicity values obtained from long-term tests are compared with those obtained from routine bioassays. It was found that certain wastes may cause chronic or cumulative toxicity.

Ludzack, Ingram, and Ettinger (1957) studied the characteristics of a stream composed of oil refinery and activated sludge effluents. They found adverse effects on aquatic organisms, including fish, for about 30 miles below the outfall. Clemens and Crawford (mimeo., no date) studied the toxicity of Oklahoma refinery effluents to red shiners

and goldfish in 1954-55. They found that only a few of the effluents studied were nontoxic in 96-hour tests and that the majority had a median tolerance limit (TL_m)[1] of less than 30 percent.

Turnbull, DeMann, and Weston (1954) reported the results of short-term bioassay investigations conducted since 1935 at the Waste Control Laboratory of the Atlantic Refining Company. The toxicity to fish of wastes from various processing units and composite wastes was compared. Additional tests were made on the toxicity of pure compounds and industrial products that may be present in a refinery effluent. Routine toxicity experiments were made by Wallen, Greer, and Lasater (1957) on 86 individual chemicals that may occur in refinery wastes. A list of 55 additional chemicals that are known to be potential components of refinery wastes was presented.

The Oklahoma Oil Refiners' Waste Control Council and the Aquatic Biology Laboratory of the Zoology Department at Oklahoma State University have cooperated in studies of refinery effluents since 1957. Monthly toxicity bioassays and chemical analyses were made on effluents of 14 refineries (Dorris, Gould, and Jenkins, 1959). Some related projects, completed or currently under investigation, are studies on toxicity changes of stored refinery effluents (Gould and Dorris, 1961), the biological and chemical changes in waste stabilization ponds (Dorris, Copeland, and Patterson, 1961), the interaction of toxic components of wastes, and the relative resistance of various species of fish to refinery effluents. The investigation reported herein is a part of the

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[1]The median tolerance limit (TL_m) is defined as the concentration of test material at which 50 percent of the test animals survive for a specified period of exposure - 24, 48 or 96 hours.

program of determining the effect of refinery effluents on aquatic life and of evaluating the effectiveness of waste treatment methods. The study was supported by National Institutes of Health Research Grant RG-6407.

MATERIALS AND METHODS

All experiments were conducted in a laboratory maintained at a temperature near 25 C. Effluents, test fish and dilution water were usually stored in the same laboratory. Temperature of the test solutions was maintained at 22 to 24 C., with an extreme range of 21 to 26 C.

Dilution Water

Stillwater, Oklahoma, tap water was used as the diluent for preparing test solutions and for acclimating test specimens. The water was held in 600-gallon redwood vats for several days to allow it to adjust to laboratory temperature. It was aerated during storage to remove chlorine and to insure saturation of dissolved oxygen.

Personnel at the water treatment plant made daily chemical analyses of the water as it left the plant. During the period of this study hardness extremes were 138 to 153 ppm, bicarbonate alkalinity ranged from 107 to 132 ppm, and carbonates were 0.0 ppm. A more complete analysis made by the U.S. Geological Survey in July, 1961, is given in Table I.

The pH of the diluent water used for each test ranged from 7.8 to 8.4. Total alkalinity for most diluent waters ranged from 108 to 118 ppm.

TABLE I

CHEMICAL CHARACTERISTICS OF DILUTION WATER

Material	ppm	Material	ppm
Calcium	37.0	Chloride	39.0
Fluoride	1.1	Nitrate	0.8
Iron	0.0	Sulfate	26.0
Magnesium	12.0	Bicarbonate	134.0
Potassium	25.0	Carbonate	0.0
Silica	3.6	Dissolved solids	218.0
Sodium	58.8	Hardness	142.0

Experimental Fish

Fathead minnows (Pimephales promelus Rafinesque) reared in farm ponds near the laboratory, were used as test animals. This species was selected because of its suitable size and adaptability to laboratory conditions. Douglas (1961) and Douglas and Irwin (1962) found it to be moderately sensitive to oil refinery wastes.

All fish used in a test were from the same collection and a new collection was made for each effluent tested. Specimens were held in the water used in transportation until it had adjusted to the laboratory temperature. Fish were then transferred to 40-gallon porcelanized tanks containing aerated tap water and held for 8 to 10 days before use in bioassay. Water in the holding tanks was treated with terramycin as described by Irwin (1959) to control bacterial infection. Heavy mortality of fish occurred only during the summer and

was generally limited to the first day after capture. Fish were not used when the incidence of disease or mortality exceeded 10 percent during the holding period. Fish were fed a mixture of ground poultry pellets and meat meal during the acclimation period. They were fed live Daphnia as a supplement when held for extended periods.

Specimens used for testing were carefully sorted to size and condition. Fish used for most tests ranged from 42 to 51 millimeters in total length. Slightly larger specimens were used for some winter and spring tests when sufficient numbers of the desired size were difficult to obtain. The range in length of individuals for a single test was usually less than six millimeters. Examination of the gonads of several hundred fish during spawning season showed that less than 15 percent of specimens about 45 millimeters long were mature females. Breeding males could easily be recognized and discarded.

Effluents

Wastes from four refineries were studied so that variation in toxicity due to differences in refining processes and waste treatment methods could be considered. All samples used were total effluents, consisting of combined wastes from various processing units.

A brief description of refinery operations and waste treatment methods is given below. Letters used to identify refineries correspond to those used in the tables and text.

Refinery A. Operations consisted of crude and vacuum distillation, catalytic and thermal cracking, sulfuric acid alkylation, polymerization, isomerization, catalytic reforming, and gasoline treating. Samples were taken after the effluent had been treated by skimming, aeration,

and final settling in ponds. Sour waters were stripped independently before being combined with other waters.

Refinery B. Processing consisted of crude and vacuum distillation, catalytic cracking and reforming, and oil blending. Samples were taken after the waste had been treated by oil separation, sour-water stripping, reuse of processing water in the desalter, flue gas neutralization of caustic solutions low in cresylic acids, removal and sale of caustic solutions high in cresylic acids, and settling in a holding basin.

Refinery C. Processing included crude and vacuum distillation, catalytic cracking, H.F. alkylation, propane deasphalting, and catalytic reforming. Samples of the waste water were taken after passage through a series of three oil settling ponds, an aeration system, and a series of four oxidation ponds. The total holding time of the ponds was 60 days.

Refinery D. Operations included atmospheric and vacuum crude distillation, solvent treating and dewaxing of lubricating oils, wax pressing and sweating, oil and grease blending, thermal and catalytic cracking, catalytic reforming and polymerization, H.F. alkylation, aromatic extraction, delayed coking, gasoline and distillate treating, and cooling tower and boiler feed water treating. Samples were from effluent that had been treated by monitoring and segregation of waste waters, removal and sale of caustic solutions rich in acid oils, impoundment of other strong caustic solutions in open pits, steam stripping of sour water from cracking operations, and an activated sludge pond. Outflow from this pond passed through 2 miles of open ditch to a cement pond from which it was pumped into a series of 10 oxidation ponds.

Effluent samples from refineries A and B had received only partial treatment and were of relatively high toxicity. They required considerable dilution to obtain survival of test fish in the routine bioassay. Samples from refineries C and D had received more extensive treatment and were of relatively low toxicity. They required little or no dilution. The objective of the sampling design was to compare long-term effects of more toxic wastes with effects of treated effluents of low toxicity.

The refineries sent one or more samples of waste water to the Aquatic Biology Laboratory for routine toxicity bioassay each month. Chemical analyses of the samples were made by refinery chemists. Wide variations occurred in toxicity and chemical composition of the effluents (Table II). The data from refineries A and B are for waste samples collected at the same place where samples were obtained for long-term bioassays. Information from refineries C and D is for samples taken at the ends of the holding pond series, but only part of the wastes studied in the long-term bioassays were collected there.

Effluents used in continuous-flow bioassays were collected and stored in 5-gallon polyethylene bottles. The bottles were filled to the top and tightly capped to minimize exposure to air. Effluents were stored in the constant temperature room during warm seasons and in a cooler room during winter. Samples were analyzed at the laboratory for ammonia, phenol, sulfide, alkalinity and pH within 1 to 3 days after collection.

TABLE II

TOXICITY AND CHEMICAL CHARACTERISTICS OF EFFLUENTS FROM FOUR REFINERIES, 1960, 61, 62

Refinery (No. of Samples)		TL_m^{48} Percent	pH	Alkalinity P[a] ppm	Alkalinity Total ppm	Ammonia as N ppm	Phenol ppm	Sulfides ppm	COD[b] ppm
A	Range	4-61	6.2-10.4	0-389	105-483	14-340	0.4-15	0-290	28-268
(19)	Mean	19		167	261	107	5.6	17.6	149
B	Range	4-70	8.8-10.6	70-300	160-855	6-110	2.3-50	0-7.8	236-525
(20)	Mean	21		166	356	64.3	14.8	0.5	352
C	Range	----	8.0-9.1	0-32	64-156	0.7-15	0.1-0.8	0-0	92-240
(20)	Mean	GT 100[c]		13	105	8.8	0.3	0	119
D	Range	----	6.8-8.7	0-22	96-106	7-43	0.1-3.1	0-2.4	112-254
(19)	Mean	GT 100		6	122	17	1	0.7	182

[a] P = Phenolphthalein alkalinity
[b] COD = Chemical oxygen demand
[c] GT = Greater than

Bioassays

Procedures used for the short-term or routine bioassay closely followed those suggested by Doudoroff, et al. (1951), and Henderson and Tarzwell (1957). Test containers were cylindrical glass jars of 5-gallon capacity. Ten fish were placed in each jar with 10 liters of experimental solution. Duplicates were prepared so 20 specimens were used for each test concentration.

Renewal of test solutions has been suggested as a modification of the routine bioassay (Doudoroff, et al. 1951; APHA, 1960). Test fish would be exposed to more uniform concentrations of toxic effluent components and metabolic products would be removed. The renewal procedure was used for long-term tests since routine bioassays indicated that a gradual reduction occurred in toxicity of refinery effluents after dilution water and fish were added. A constant-flow apparatus was constructed to continuously renew test solutions (Figure 1).

Metering pumps[1] were used to regulate the flow of effluent and dilution water (Figure 2). Pumps were constructed of cutlery grade stainless steel with internal surfaces highly finished and corrosion resistant. Delivery rates of pumps used in tests are given in Table III. Only five pump sizes were available but a sixth delivery rate (3x) was obtained by using a different gear combination with a size 3 pump. All pumps used in a test were driven by a common shaft turned by a motor with a variable speed transmission (Figure 2). Since effluent and dilution water flows were regulated by the same equipment, any change in the power supply affected all units equally.

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[1]The metering pumps were designed to spin rayon yarn and were manufactured by Zenith Products Company, West Newton, Massachusetts.

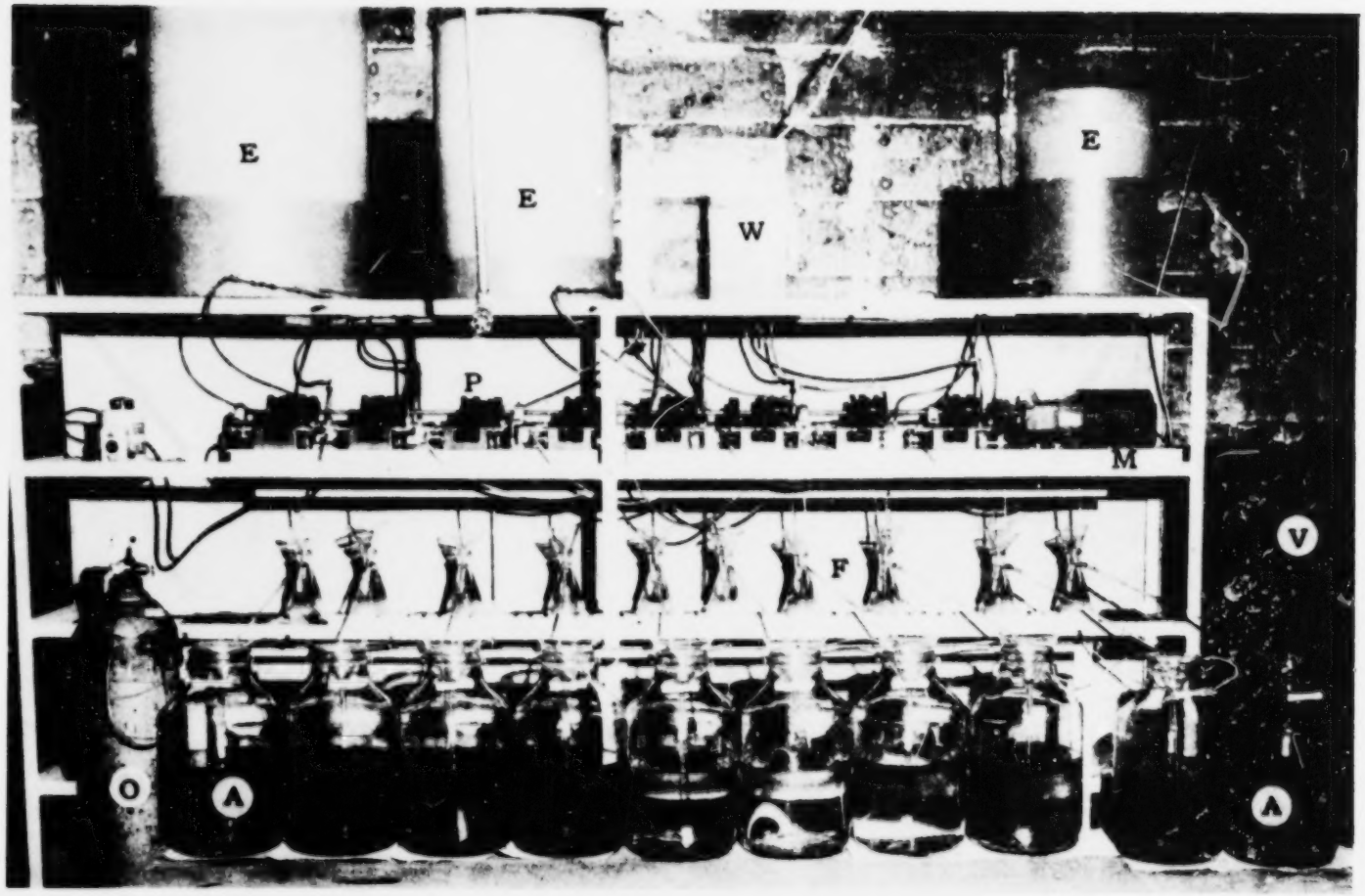

Figure 1. Constant-flow apparatus used in long-term bioassay of oil refinery effluents: effluent (E), water (W), water-holding tanks (V), pumps (P), motor (M), mixing flasks (F), aquaria (A), and oxygen (O).

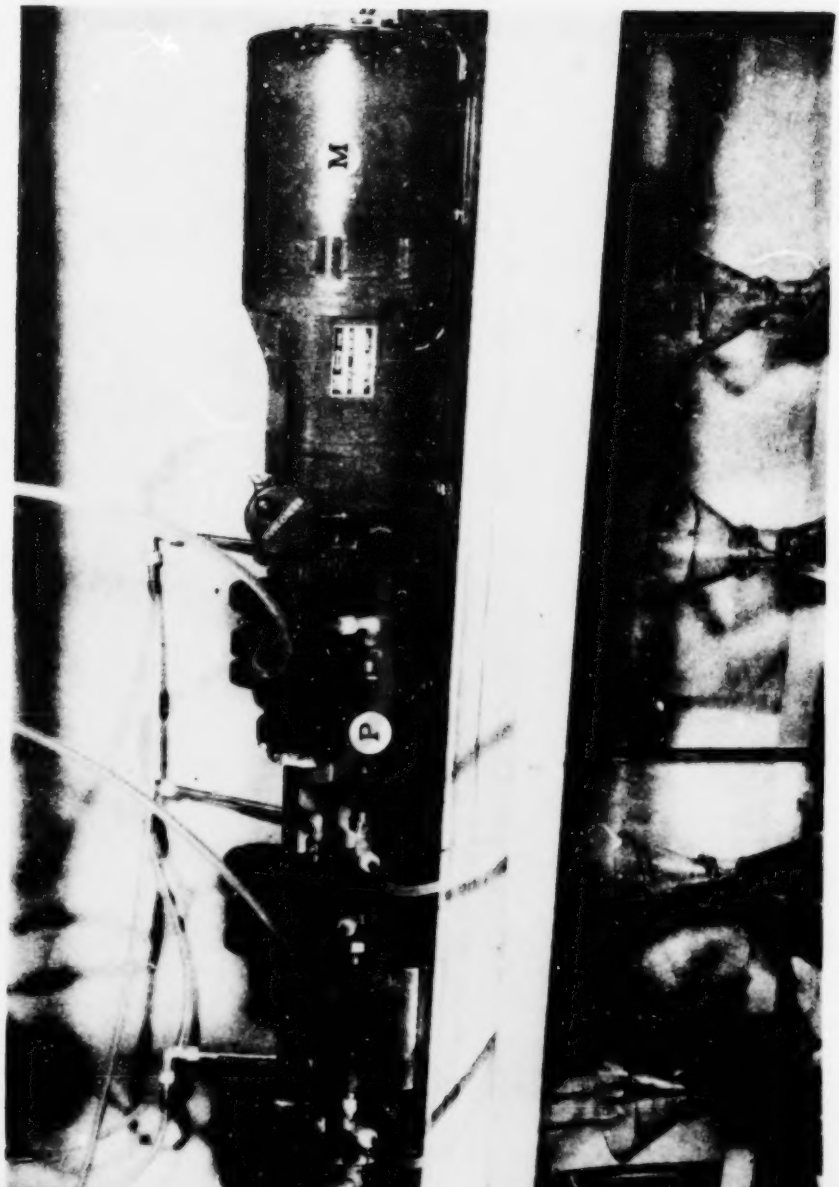

Figure 2. Pumps (P) and motor (M) used in delivering water and effluent to test aquaria.

TABLE III

METERING PUMP DELIVERY RATES AND SOLUTION CONCENTRATIONS USED IN LONG-TERM BIOASSAYS

Pump Size	Delivery at 10.25 r.p.m.		Pump Combinations		Total Delivery	Effluent Concentration
	cc/min	1/24 hr	Waste Water	Dilution Water	cc/min	(percent)
½	3	4.5	½ and	5	33	9
1	6	8.7	1 and	3x	30	20
2	12	17.4	2 and	3	30	40
3	18	25.7	3 and	2	30	60
3x	24	34.4	3x and	1	30	80
5	30	43.1	5		30	100

Solution concentrations obtainable at a given shaft speed with various metering unit combinations are given in Table III. Concentrations delivered by each combination of pumps were measured during each test and the variation was never more than 0.5 percent. At 10.25 r.p.m., the pumps delivered solutions to test aquaria at about 30 cc/min. This flow theoretically replaced 10 liters of solution in each aquarium every 5.5 hours. A more rapid replacement rate would have kept the composition of test solutions more uniform but was not practicable because of the large quantities of effluent needed for prolonged tests.

Although pumps were made of stainless steel parts in contact with the metered fluid, particularly dilution water, became slightly corroded. Since the working surfaces were finished to a tolerance of 0.000025 inch, a slight amount of rust could cause a pump to jam. Pumps of size ½, 1, and 2 could be operated safely for 15 days without cleaning but sizes

3 to 5 had to be cleaned after 8 to 10 days operation. Working surfaces were cleaned with alcohol and lubricated with a thin film of castor oil. Pumps were flushed with water for 10 to 15 minutes before use in a test to remove excess oil.

Dilution water was pumped from the holding vats (V, Figure 1) to a plexiglass constant head box (W). Effluent was held in polyethylene barrels (E) with capacities just sufficient to supply liquids for about 12 hours operation. Five-gallon barrels were used for high-toxicity effluents and 55-gallon containers for less toxic wastes. By decreasing the liquid surface area/volume ratio the escape rate of volatile components was diminished. Dilution water and effluent were pumped from the containers to 500 cc suction flasks (F) where they mixed. Solutions overflowed from mixing flasks to test aquaria (A). The volume of solution in a test container was maintained at 10 liters by an overflow located about 7 inches above the container bottom. Long-stemmed funnels were used in the mixing flasks and test aquaria so that inflowing liquids were introduced near the bottom. Connections and tubes through which liquids passed were stainless steel, polyethylene, nylon, or glass.

Exploratory bioassays were conducted on effluents the day after their collection. Solutions were prepared covering a wide range of concentrations. Five test fish were placed in 5 liters of the solutions for about 24 hours. Test results indicated the concentrations to be selected for full scale bioassays.

Solution concentrations selected for both routine and long-term bioassays were limited to those obtainable with the continuous-flow apparatus. For a few long-term tests, concentrations less than 9 percent were obtained by prediluting effluent with distilled water.

Only concentrations in which more than 50 percent of the test animals survived in the routine test were used for long-term tests. The concentrations are called "sub-acute" in this study. Controls were established for each routine and long-term test using dilution water as the test solution.

The apparatus could not be assembled until results from the exploratory bioassays were obtained. Since assembly required several hours, full scale tests were not begun until 2 to 3 days after the effluents were collected. Solutions for the routine and long-term tests were prepared simultaneously. Measurements were made of pump delivery rates and of the oxygen content and pH of the solutions before fish were added.

Test solutions having an initial dissolved oxygen concentration less than 2.0 ppm were treated with oxygen before fish were added. Oxygen was bubbled slowly into the test solutions if the dissolved oxygen content became reduced to about 2.0 ppm (0, Figure 1). Dissolved oxygen content was maintained at higher levels when free carbon dioxide accumulated in the test solutions. The Alsterberg (sodium azide) modification of the Winkler method was used for dissolved oxygen measurements. Free carbon dioxide was measured by titration (APHA, 1960) and the pH was determined with a Beckman meter.

Routine tests were terminated at 96 hours. An exposure period of 32 days was arbitrarily selected for the long-term bioassay. Some of the long-term tests had to be terminated before 32 days because of equipment failure or poor survival of control fish. To facilitate comparisons, results for such tests are reported for a 16 day period. Fish were not fed in routine bioassays. In long-term tests, feeding

was started on the fifth day and continued once daily *ad libitum* with live *Daphnia*.

In some tests, fish surviving long-term exposure to effluent solutions were compared to fish in corresponding controls by means of a condition index, K (Carlander, 1953):

$$K = \frac{\text{Weight in grams} \times 100{,}000}{(\text{Total length in millimeters})^3}.$$

The condition index is often used to describe changes in body form or well-being of fish. It is acceptable here because fish of comparable length were selected for each test and lots of fish were randomly assigned to test solution and control aquaria. Comparison of condition indices between experiments is not valid since condition normally varies with size and age of the individuals.

In addition to recording casualties at daily intervals, notes were made on fish behavior. When introduced into test solutions fish went to the bottom and swam around the aquarium periphery in a school. This characteristic persisted throughout the tests with fish in control aquaria. Control fish also responded rapidly when disturbed by rapping on the containers. Fish became distressed in nearly all effluent solutions within minutes after introduction. When distressed the fish did not school and swam around the container at various levels. Distressed fish movements were sluggish and response to disturbance was slow or absent. Severe distress was indicated by erratic swimming, darkening of the integument, periods of immobility and paralytic spasms. When the latter conditions were observed, death generally occurred within a few hours.

RESULTS

Bioassays of High-Toxicity Effluents

Seven experiments were conducted on effluents of relatively high initial toxicity. Three continuous renewal tests were 16 days long and four were 32 days. The 96-hour TL_m from routine tests ranged from 6.5 to 16.5 percent. High-toxicity effluents did not contain large numbers of planktonic organisms compared to the more extensively treated low-toxicity effluents. Bacterial slime growths formed in the mixing flasks and tubing through which the effluent solution flowed and had to be removed every 6 to 8 days to prevent stoppage of flow. A thin gelatinous growth formed on the walls of test aquaria. Clearing of test aquaria was limited to siphoning fish excrement and unused food from the bottoms.

Dissolved oxygen concentration and pH of routine bioassay test solutions were determined at the beginning of each experiment and when all fish had died or after 96 hours (Table IV). In long-term tests oxygen content and pH were recorded at 4-day intervals. Oxygen determinations were made frequently to insure the oxygen levels to be 2 ppm or higher.

Oxygen concentration and pH decreased in test solutions in both testing methods. Reduction at comparable test concentrations was greater in routine tests than in long-term tests, showing that continuous renewal of test solutions maintained relatively constant

TABLE IV

DISSOLVED OXYGEN CONTENT AND pH OF TEST SOLUTIONS DURING BIOASSAY OF HIGH-TOXICITY EFFLUENTS

Test Number	Percent Effluent	Routine Test				Continuous Renewal Test		
		pH		DO[a] (ppm)		pH	DO[a] (ppm)	
		Init.	End	Init.	End	Range	Avg.	Range
1	20	8.5	8.0	6.8	3.2			
	9	8.4	7.5	7.4	5.4	7.7-8.4	4.8	3.3-7.6
	Control	7.9	7.6	7.6	6.0	7.8-8.1		
2	20	8.5	8.5	6.8	6.4			
	9	8.4	7.7	7.2	1.9	7.9-8.5	5.9	4.8-7.8
	Control	8.1	7.6			7.9-8.1		
3	20	8.2	8.1	5.8	5.0			
	9	8.1	7.5	6.8	4.0	7.8-8.1	5.3	4.2-7.2
	Control	7.9	7.5			7.8-7.9		
4	20	8.7	7.6	4.1	3.8[b]			
	9	8.6	7.7	6.0	4.7[b]	7.8-8.6	3.3	2.2-6.2
	4.5					7.7-8.3	4.7	3.5-7.0
	Control	8.1	7.5			7.8-8.1		
5	20	8.4	8.4	3.2	2.7			
	9	8.3	7.5	4.4	2.5[b]	8.3-7.6	2.9	1.9-5.9
	4.5					8.3-7.6	3.8	2.3-6.4
	Control	8.1	7.5			7.8-8.1		
6	9	8.7	8.7	6.5	5.0			
	4.5	8.6	7.6	7.0	1.9	7.8-8.6	3.7	2.5-7.2
	2.2					7.7-8.5	4.8	3.2-7.6
	Control	8.0	7.5			7.9-8.0		
7	20	8.7	8.6	7.0				
	9	8.6	7.5	7.6	7.0[b]	7.8-8.6	2.4	1.9-8.4
	4.5					7.7-8.5	2.8	2.3-8.6
	Control	8.1	7.7			7.9-8.1		

[a] Dissolved oxygen.
[b] Oxygenated during the test.

conditions. At the highest solution concentrations in most routine tests, all fish died shortly after the tests began. Oxygen content and pH were measured at that time and had decreased only slightly.

Fish survived extended exposure to sub-acute concentrations of high-toxicity effluents. Survival during 96 hours was about the same when test solutions were renewed as when not renewed (Table V). Fish survived 16 days exposure in all but one of the experiments. In Experiment 1, 35 percent mortality occurred by the 16th day at the sub-acute effluent concentration of 9 percent, but all fish survived the concentration in the routine test. Survival after 32 days exposure was also comparable to survival in routine tests.

Surviving fish did not appear to suffer adverse effects in sub-acute concentrations of high-toxicity effluents. During the long-term tests no differences in behavior or appearance were detected between specimens in the effluent solutions and those in the control solutions. Condition factors were determined for surviving fish in three of the 32 day experiments (Table V). Mean condition factors of fish in effluent solutions were not significantly different from those of fish in the controls.

Changes in Stored High-Toxicity Effluents

Chemical characteristics of effluents at the beginning of each experiment and for some tests, after 16 or 32 days of storage, are given in Table VI. In general, there was a decrease in concentrations of ammonia, phenol, and sulfide during storage. The greatest change occurred in the effluent of Experiment 6 where ammonia content decreased from 132 to 75 ppm in 32 days. Increase in sulfide content in the

TABLE V

FISH SURVIVAL IN ROUTINE AND LONG-TERM TOXICITY BIOASSAYS
OF EFFLUENTS WITH INITIAL HIGH TOXICITY

Experiment Number	Refinery	Date Test Began	Percent Effluent	Routine Test Percent Survival 96 hr	Continuous Renewal Test			
					Percent Survival			Mean Condition Factors
					96 hr	16 day	32 day	
1	A	8-15-60	20	0				
			9	100	100	65		
			Cont.	100	100	100		
2	B	9-12-61	20	0				
			9	100	95	95		
			Cont.	100	100	95		
3	B	10-3-61	20	0				
			9	100	100	100		
			Cont.	100	100	95		
4	B	9-20-60	20	35				
			9	100	100	100	100	0.64
			4.5		100	95	90	
			Cont.	100	100	100	100	0.61
5	B	11-12-60	20	0				
			9	95	100	95	80[a]	0.70
			4.5		100	100	100	0.75
			Cont.	100	100	100	100	0.73

[a]One fish became entrapped in funnel stem and died.

TABLE V (Continued)

Experiment Number	Refinery	Date Test Began	Percent Effluent	Routine Test Percent Survival 96 hr	Continuous Renewal Test			
					Percent Survival			Mean Condition Factors
					96 hr	16 day	32 day	
6	A	6-8-61	9	0				
			4.5	100	100	95	85	0.67
			2.2		100	100	85	0.68
			Cont.	100	100	100	90	0.71
7	A	7-18-61	20	0				
			9		100	95	95	
			4.5	100	100	100	95	
			Cont.	100	100	100	100	

[a]One fish became entrapped in funnel stem and died.

TABLE VI

CHEMICAL CHARACTERISTICS OF HIGH-TOXICITY EFFLUENTS AT THE BEGINNING OF EXPERIMENTS AND AT THE END OF THE STORAGE PERIOD

Test Number	Days Stored	Time of Analysis	pH	Alkalinity		Ammonia ppm	Phenol ppm	Sulfide ppm
				P[a] ppm	Total ppm			
1	16	Initial	8.7	75	165	78	11.4	1.1
		End	8.7	55	165	68	11.2	0.4
2	16	Initial	8.8	126	232	60	6.0	0.0
		End	8.7	-	-	-	-	-
3	16	Initial	8.7	112	192	88	6.8	0.0
		End	8.5	52	120	70	4.2	0.0
4	32	Initial	8.6	65	160	44	21.2	9.2
		End	8.5	55	165	40	20.0	6.2
5	32	Initial	8.7	85	238	44	19.8	17.6
		End	8.6	80	195	40	16.0	25.6
6	32	Initial	9.3	150	360	132	21.3	0.95
		End	9.2	150	300	75	13.0	Trace
7	32	Initial	9.2	108	176	78	10.5	Trace
		End	8.9	-	-	-	-	-

[a]P = Phenolphthalein alkalinity

effluent of Experiment 5 may have been due to analytical error. A slight decrease in pH occurred in most effluents during storage.

Routine toxicity tests were made on each effluent at intervals throughout the storage period to measure changes in toxicity (Table VII). Only one of the seven effluents decreased in toxicity during 16 days of storage (96-hour exposure). In the effluent of Experiment 1, survival at 20 percent effluent concentration increased from zero to 15 percent. Two of the four effluents decreased in toxicity during 32 days of storage. The greatest change occurred in effluent of Experiment 7 where survival increased from 0 to 65 percent. Gould and Dorris (1961) studied toxicity changes of two effluents with initial high-toxicity. They found no significant change in 96-hour TL_m values during 30 days of storage.

Although the 96-hour results showed that toxicity changes occurred in only three of the seven stored effluents, the time it took to kill fish during routine tests indicates that they all decreased in toxicity. After effluents had been stored, survival at the 1 or 6 hour period of the routine tests increased.

The decrease in toxicity during storage, indicated by analytical and biological procedures, was considered serious enough to invalidate results of the long-term tests. Effluents remained very toxic during storage and sub-acute concentrations used in the long-term tests were near acute toxicity concentrations.

It should be noted that 24 hour and 96 hour survival values were nearly identical (Table VII) showing that most mortality in the routine tests occurred within 24 hours. This suggests a gradual reduction in toxicity of the test solutions.

TABLE VII

ROUTINE TOXICITY TESTS OF EFFLUENTS
DURING STORAGE

Experiment Number	Percent Effluent	Time of Test	Percent Survival			
			1 hr	6 hr	24 hr	96 hr
1	20	Initial	100	25	0	0
		16 day	95	65	30	15
2	20	Initial	30	0	0	0
		16 day	40	5	0	0
3	20	Initial	5	0	0	0
		16 day	60	10	0	0
4	20	Initial	100	75	35	35
		16 day	100	65	40	40
		32 day	100	85	50	50
5	20	Initial	0	0	0	0
		16 day	75	0	0	0
		32 day	80	0	0	0
6	9	Initial	45	0	0	0
		16 day	70	10	0	0
		32 day	75	25	0	0
7	20	Initial	90	0	0	0
		16 day	95	0	0	0
		32 day	100	95	65	65

Bioassay of Low-Toxicity Effluents

Eleven experiments were made on effluents with initial low toxicity. Four of the continuous renewal tests were 16 days long, one was 24 days long and 6 were 32 days long. The TL_m^{96} of effluents used in three experiments ranged from 50 to 72 percent. In eight experiments there were few to no deaths in 96 hours at effluent concentrations of 100 percent (Table X).

Large quantities of low-toxicity effluents were needed to complete prolonged tests. For two 16 day experiments (8 and 10) one collection of effluent was sufficient for each test. Two to six collections were needed for each of the other experiments. The number of collections made and chemical characteristics of the effluents used for each experiment are presented in Table VIII. Concentrations of ammonia, phenol and sulfides in the extensively treated wastes were much less than in the high-toxicity effluents (Table VI).

Toxicity and chemical characteristics of each new collection of effluent were determined before it was used in the test. If a new batch of effluent varied greatly from the original collection, particularly with respect to toxicity, the test was discontinued.

Effluents generally contained greater numbers of planktonic organisms than the more toxic wastes. This was especially true for spring and summer samples when large blooms of green algae occurred in the waste stabilization ponds. Formation of slime growths in mixing flasks and tubing of the continuous flow apparatus was also greater than for the high-toxicity effluents. Equipment frequently had to be cleaned every 3 to 4 days. Biogrowths and dead algae accumulated on the walls

TABLE VIII

CHEMICAL CHARACTERISTICS OF LOW-TOXICITY EFFLUENTS

Test	Refinery	Number of Batches	pH	Alkalinity P[a] ppm	Alkalinity Total ppm	Ammonia as N ppm	Phenol ppm	Sulfides ppm
8	D	1	8.4	45	169	19	1.0	0.5
9	D	2	7.4-7.5	0	114-120	16-17	0	0.1-0.2
10	C	1	7.5	0	161	19	0.7	Trace
11	C	2	7.3-7.4	0-46	144-146	20-22	1.6-3.6	0
12	C	4	7.8-8.0	0	142-148	14-18	0.1	0
13	C	4	8.0-8.5	0-22	136-150	13-16	0.4-0.5	0
14	C	4	8.3-8.8	4-22	88-120	9-13	0.1-0.3	0
15	C	3	7.1-7.4	0	93-105	6-10	0.5-2.3	Trace
16	D	6	7.2-8.0	0	74-111	7-23	Trace	0
17	D	6	7.0-7.8	0	90-103	15-24	Trace	0
18	D	4	7.4-7.5	0	100-118	0-6	0	0

[a]P = Phenolphthalein

and bottoms of the test aquaria. The materials, along with excrement and unused food, were removed periodically by siphoning. In Experiments 13, 14, 16 and 17 fish were transferred to clean aquaria at the sixteenth day.

Artificial oxygenation was necessary for nearly all test solutions in both routine and long-term bioassays. Since low-toxicity effluents required little dilution to obtain sub-acute levels, the concentrations of organic matter and respiring organisms in test solutions remained high. Oxygen demand was consequently higher than in the extensively diluted, high-toxicity effluents. For similar reasons the carbon dioxide content in test solutions of low-toxicity effluents was greater than in those with high toxicity. Carbon dioxide accumulated in the storage containers as well as in the test solutions. Largest accumulations occurred when the effluents contained an abundance of algae.

For most test solutions dissolved oxygen concentration was maintained at from 7 to 9 ppm when the amount of carbon dioxide was near or greater than 10 ppm (Table IX) to minimize the interference of carbon dioxide with fish respiration. Desired oxygen levels were maintained approximately by adjusting the delivery rate (bubbles per minute) as indicated by frequent dissolved oxygen determinations. The extremes in oxygen concentration shown in Table IX generally occurred during the first several days of the experiment.

Sampling locations, test conditions and effects varied between tests therefore results are presented by individual or pairs of experiments.

TABLE IX

DISSOLVED OXYGEN, CARBON DIOXIDE, AND pH
OF TEST SOLUTIONS IN EXPERIMENTS ON
LOW-TOXICITY EFFLUENTS

Test	Percent Effluent	Continuous Flow				
		pH	Dissolved Oxygen ppm		Carbon Dioxide ppm	
		Range	Avg	Range	Avg	Range
8	60	7.6-8.3	3.7	1.8-5.7	Less than 10	
9	100	7.0-7.4	8.0	3.4-10.1	21	15-26
	80	7.1-7.6	8.8	4.2-10.0	17	12-21
10	40	7.5-7.7	4.0	2.6-8.3	Less than 10	
11	60	7.2-7.5	3.5	1.9-6.8	12	7-15
	40	7.3-7.6	4.0	1.9-7.6	10	5-12
12	100	7.2-8.0	7.3	1.9-10.2	16	0-23
	80	7.3-8.0	7.1	2.0-10.0	14	0-20
13	100	7.2-8.0	7.6	3.5-10.0	14	0-19
	80	7.3-8.0	7.4	3.8-9.8	12	0-17
14	100	7.3-8.8	7.4	2.6-10.1	12	0-14
	80	7.4-8.4	7.3	3.4-9.9	9	0-12
15	100	7.1-7.4	3.8	1.7-5.9	16	4-22
16	100	7.2-7.5	7.9	2.0-10.0	18	12-22
	80	7.2-7.6	8.0	2.2-10.1	16	11-20
	60	7.3-7.7	8.1	2.4-10.3	13	8-17
	40	7.4-7.8	7.9	3.4-9.8	11	7-13
17a	100	7.1-7.5	3.3	1.8-5.7	16	13-18
	60	7.4-7.8	3.1	1.7-6.0	11	8-13
17b	100	7.1-7.5	7.4	4.2-10.2	16	13-17
	60	7.4-7.8	7.6	5.1-10.1	11	8-13
18	100	7.3-7.5	8.8	1.9-10.1	12	8-17
	80	7.4-7.6	9.1	2.5-10.3	11	8-16
	60	7.5-7.8	8.9	2.7-9.8	9	6-13
	40	7.6-7.9	9.0	3.0-10.0	7	4-10

Experiments 8 and 9. Effluent for Experiment 8 was collected at refinery D from the cement pond while for Experiment 9, collections were made at the pond series outlet. Algae were abundant in the latter effluent and carbon dioxide accumulated during storage. The initial sample contained 4 ppm carbon dioxide when brought to the laboratory. The amount increased to 14 ppm by the second day of storage at room temperature and to 23 ppm by the eighth day. In a sample stored 8 days at 5° C the carbon dioxide content increased to 18 ppm.

Effluent for Experiment 8 was stored 16 days during which time the 96-hour TL_m increased from 67 to 69 percent. A slight loss of toxicity was indicated by a decrease in ammonia from 19 to 17 ppm and in phenol from 1.0 to 0.0 ppm. Toxicity changes during storage (about 8 days) of effluents for Experiment 9 were not measured.

In the routine test of Experiment 8, fish were distressed for about 12 hours at 60 percent concentration. Fifteen percent mortality occurred during the period (Table X). Fish were moderately distressed throughout the long term test at the concentration. Food was consumed but not as actively as in the controls. Extensive mortality began after the fifth day (Figure 3). Thirty percent of the fish survived 16 days exposure but were emaciated.

Mortality was low in 100 percent effluent in the routine tests of Experiment 9 but fish were distressed for about 10 hours (Table X). In the continuous renewal test the distress period lasted for 36 hours at 100 percent concentration and 24 hours at 80 percent. All fish in both concentrations became moderately distressed on the tenth day and the behavior persisted for the remainder of the experiment. Deaths at 100 percent concentration began on the tenth day (Figure 3) and at

TABLE X

FISH SURVIVAL IN ROUTINE AND LONG-TERM TOXICITY BIOASSAYS
OF EFFLUENTS WITH INITIAL LOW TOXICITY

Experiment Number	Refinery	Date Test Began	Percent Effluent	Routine Test Percent Survival 96 hr	Continuous Renewal Test			
					Percent Survival 96 hr	16 day	32 day	Mean Condition Factors
8	D	12-30-60	80	0	-	-		
			60	85	95	30		
			Control	100	100	100		
9	D	1-11-62	100	95-100	100	25		
			80	-	100	65		
			Control	95-100	100	100		
10	C	4-18-61	80	0	-	-		
			60	50	-	-		
			40	100	100	75		
			Control	100	100	100		
11	C	10-31-61	80	0-10	-	-		
			60	90-95	15	0		
			40	-	95	40		
			Control	100	100	95		
12	C	12-9-61	100	95-100	85	45	10	
			80	-	95	70	35	
			Control	100	100	95	90	
13	C	2-5-62	100	95-100	100	100	65	0.45
			80	-	100	100	90	0.51
			Control	100	100	100	100	0.64

TABLE X (Continued)

Experiment Number	Refinery	Date Test Began	Percent Effluent	Routine Test Percent Survival 96 hr	Continuous Renewal Test			
					Percent Survival			Mean Condition Factors
					96 hr	16 day	32 day	
14	C	2- 7-62	100	95-100	95	95	95	0.56
			80	-	100	100	100	0.57
			Control	100	100	100	100	0.64
15	C	3- 9-61	100	95-100	100	40	0	
			Control	100	100	100	100	
16	D	5-12-62	100	100	100	95	90	0.69
			80	-	100	100	100	0.73
			60	-	100	100	100	0.75
			40	-	100	100	95	0.77
			Control	100	100	100	100	0.82
17[a]	D	6-17-62	100	100	100	100	5	0.65 (0.53)[b]
			60	-	100	100	50	0.62 (0.52)
17[b]	D	6-17-62	100	100	100	100	20	0.57 (0.52)
			60	-	100	100	65	0.60 (0.52)
			Control	100	100	100	100	0.78
18	D	3-10-62	100	100	100	100	(100)[c]	0.72
			80	-	100	100	(100)	0.68
			60	-	100	100	(100)	0.72
			40	-	100	100	(100)	0.73
			Control	-	100	100	(100)	0.73

[a] Dissolved oxygen in test solution of 17[a] maintained at 3-4 ppm and in 17[b] at 7-8 ppm.
[b] Figures in parentheses are mean condition factors of fish at death.
[c] Test terminated at 24 days.

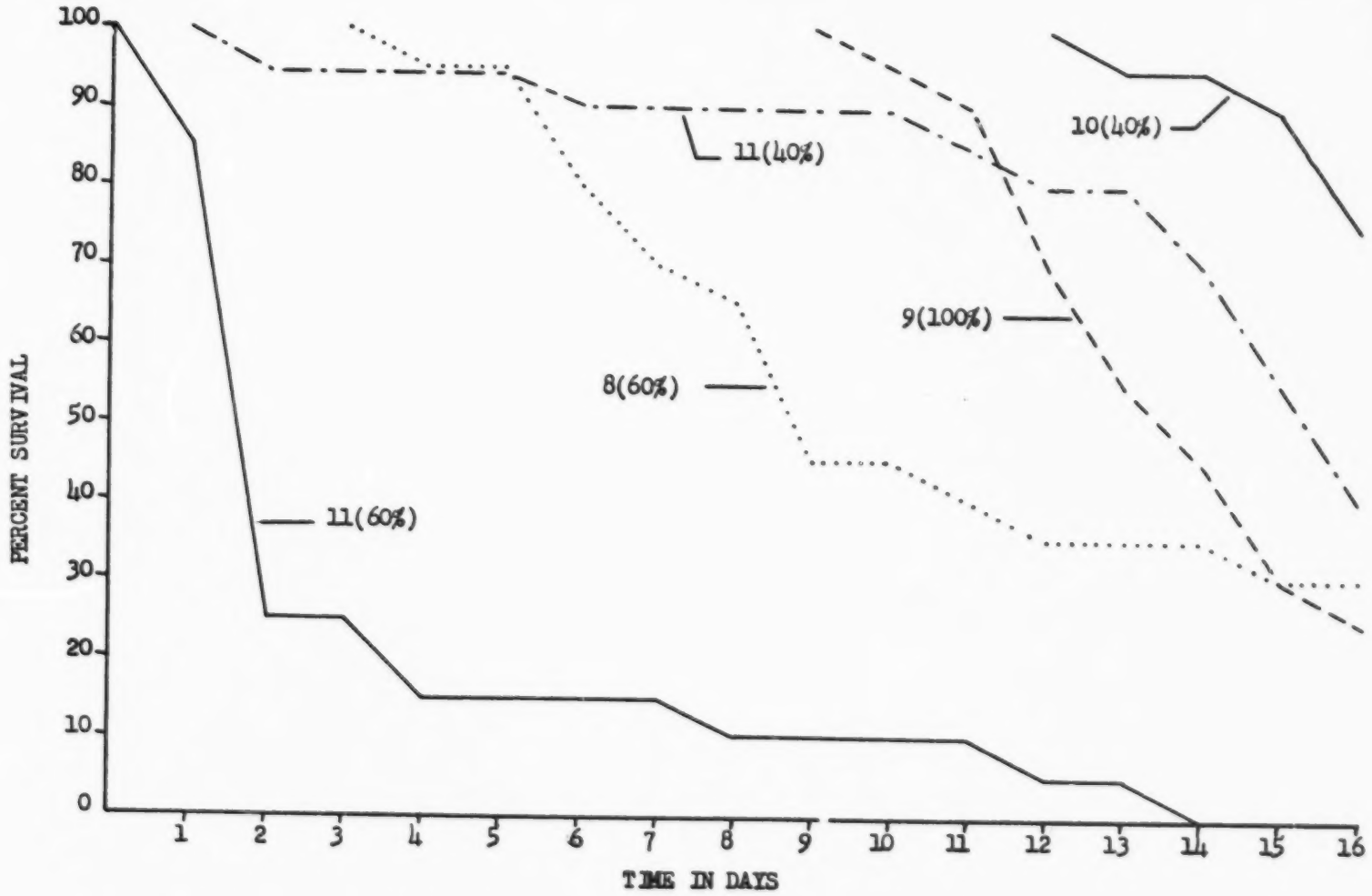

Figure 3. Survival of fish in various concentrations (parentheses) of oil refinery effluents in Experiments 8, 9, 10, and 11.

80 percent on the twelfth day. Emaciation was not apparent in either dead or surviving fish.

Experiments 10 and 11. Effluents for Experiments 10 and 11 were collected at the outlet of the third settling pond and had been held for about 16 days. Algae were not abundant in the effluents but some carbon dioxide accumulated during storage.

A single collection of effluent was made for Experiment 10. During 16 days storage ammonia content decreased from 18.5 to 10.0 ppm and phenol from 0.7 to 0.0 ppm. The 96-hour TL_m changed from 60 to 80 percent. In spite of the decrease in toxicity 25 percent mortality occurred in the long-term tests at 40 percent concentration (Table X). Fish were distressed for 12 hours in the routine test at 40 percent concentration. In the continuous renewal test fish were distressed during the first 72 hours but then appeared normal until just before death. Deaths began on the thirteenth day (Figure 3).

Two collections of effluent were needed for Experiment 11. The 96-hour TL_m of the first collection changed from 69 to 72 percent during 10 days of storage. No other measurements of storage effects were made for this experiment.

Fish were distressed for 24 hours at 60 percent concentration in the routine test. In the continuous renewal test the distress period was 48 hours and 75 percent mortality occurred (Figure 3). Surviving fish behaved normally except for a loss of appetite. Fish in the 40 percent concentration of the long-term test behaved normally by the end of the first day but became distressed again on the eighth day, and

50 percent mortality occurred between the tenth and sixteenth days (Figure 3). Condition indices were not determined but dead and surviving fish did not appear emaciated.

Experiment 12. Effluent for Experiment 12 was collected near the inlet of the third oxidation pond with an accumulated holding time of about 20 days. Algae were not abundant.

No significant mortality occurred in routine tests at 100 percent concentration (Table X). The distress period for the four effluent batches used in the experiment ranged from 3 to 6 hours. Fish in the continuous renewal test were distressed for 40 hours at 100 percent concentration and 36 hours at 80 percent. Moderate distress and loss of appetite began again in both concentrations on the seventh day and continued throughout the test. The beginning of extensive deaths coincided with the renewal of distress behavior (Figure 4). Fish that died during the first half of the experiment were in good body condition but many that died in the second half were emaciated.

Experiments 13 and 14. These experiments were run concurrently with effluents taken from different locations in the treatment system of Refinery C. Effluent collections for Experiment 13 were made near the inlet of the third oxidation pond (20 days holding time) while those for Experiment 14 were made at the end of the pond series (60 days holding time). Algae were abundant at both locations.

Four collections of effluent were needed for each experiment and storage time for individual collections was about 8 days. In Experiment 13 average ammonia content in stored effluents decreased from 15 to 12 ppm and pH values decreased about 0.4 of a unit. In Experiment 14

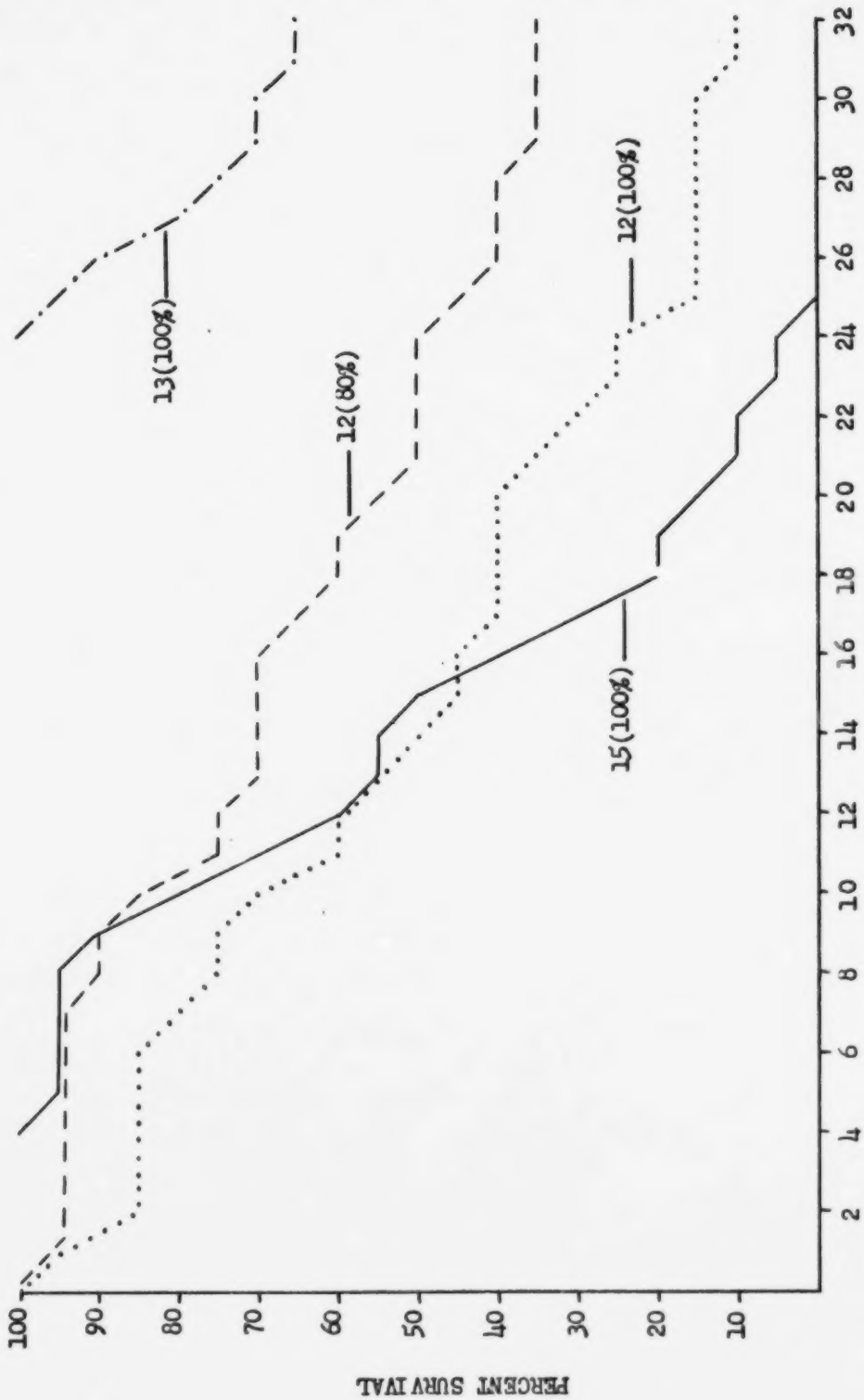

Figure 4. Survival of fish in various concentrations (parentheses) of oil refinery effluents in Experiments 12, 13, and 15.

average ammonia decreased from 11.5 to 9.5 ppm and the pH values about 0.8 of a unit. Toxicity changes during storage were not measured but the chemical analyses indicated a loss of toxicity.

Fish behavior in both experiments was the same. In routine tests the distress period in the various collections ranged from 6 to 12 hours. In continuous renewal tests the distress period lasted about 40 hours in all effluent solutions. Ensuing fish behavior and food consumption were normal but by the sixteenth day fish in test solutions of Experiment 13 were becoming emaciated. By the twentieth day fish in Experiment 14 were also emaciated. Thirty-five percent mortality at 100 percent concentration in Experiment 13 occurred during the last 8 days (Figure 4). Surviving fish in 100 and 80 percent concentrations were emaciated, swam erratically and had darkened integuments.

Condition indices were determined for dead and surviving fish in all test solutions of both experiments (Table X). Mean condition factors for fish in 100 and 80 percent solutions of Experiment 13 include the indices of dead fish. The dead fish were used because deaths occurred during the last week and reflected the effects of long exposure. Mean condition factors of dead fish were similar to those of surviving fish.

Mean condition indices were ranked and compared using Duncan's new multiple range test (Steel and Torrie, 1960). The results at the one percent confidence level using 100 as the error degrees of freedom are summarized in Table XI. Condition of fish in effluent solutions was significantly poorer than condition of the control fish. Mean condition indices were progressively less from the most treated effluent at 80 percent concentration to the least treated waste at 100 percent concentration.

TABLE XI

COMPARISON OF MEAN CONDITION INDICES FOR FISH IN EXPERIMENTS 13, 14, AND 16[a]

	Experiment 13		Experiment 14		
Effluent Concentration	100%	80%	100%	80%	Control
Mean Condition Index	0.45	0.51	0.56	0.57	0.64

	Experiment 16				
Effluent Concentration	100%	80%	60%	40%	Control
Mean Condition Index	0.69	0.73	0.75	0.77	0.82

[a]Any means underscored by the same line are not significantly different.

Experiment 15. Three effluent collections for Experiment 15 were made at the cement pond outlet. Algae were not abundant but carbon dioxide gradually accumulated in the test solutions (Table VIII). Toxicity and chemical changes during storage were not measured.

No significant mortality occurred in the routine tests at 100 percent concentration but the fish were moderately distressed for about 24 hours. In the long-term tests the fish showed moderate but gradually diminishing distress behavior for the first 6 days. Thereafter, the fish remained at the bottom of the aquaria until just prior to death but their food consumption and response to stimulus was below that of the control fish. Extensive deaths in 100 percent concentration began to occur on the ninth day and all fish were dead by the twenty-fifth day (Figure 4). Condition factors were not determined but many of the fish that died during the last 10 days of the test were emaciated.

Experiment 16. Six collections of effluent for Experiment 16 were made at the outlet of the pond series. Algae were abundant and carbon dioxide accumulated in the storage containers and test solutions (Table IX). Carbon dioxide concentration in test solutions was generally 1 to 2 ppm greater than in storage containers at the corresponding time. Oxygen concentration in the test solutions was maintained at about 8.0 ppm.

In addition to the chemical characteristics presented in Table VIII, analyses were made for other potential toxicants in each effluent collection (Table XII). Zinc was not included in the analyses because recovery procedures proved inadequate. All chemical analyses for Experiments 16 and 17 were made by refinery chemists.

No deaths occurred in routine tests of the six samples at 100 percent concentration. Distress periods in the routine tests were less than 12 hours. Fish behaved normally within 24 hours in the continuous renewal tests and deaths occurring during 32 days of exposure were 10 percent or less (Table X). It was apparent by the last week that fish in 100 percent concentration were emaciated even though they consumed food actively.

The mean condition factors of the surviving fish were compared using Duncan's 5 percent multiple range test (Table XI). Condition of fish in effluent concentrations of 100, 80, and 60 percent was significantly less than those of control fish. Condition of fish in 40 percent concentration and the controls did not differ significantly. Although differences between mean condition factors of fish in effluent solutions were not significant in every case, condition decreased progressively as effluent concentration increased.

TABLE XII

CHEMICAL COMPONENTS IN EFFLUENTS
USED FOR EXPERIMENTS
16 AND 17

	Test 16		Test 17	
	Range	Average	Range	Average
Cyanide as CN, ppm	0.0-0.008	0.002	0.0	0.0
Thiocyanate as CN, ppm	0.014-0.048	0.028	0.012-0.048	0.031
Total Chromium as CrO4, ppm	0.03-0.11	0.05	0.03-0.06	0.05
Hexavalent Chromium as CrO4, ppm	0.0	0.0	0.0	0.0
Copper as Cu, ppm	0.03-0.13	0.08	tr.-0.05	0.02
Arsenic as As, ppm	0.01-0.01	0.01	0.003-0.011	0.006
Lead as Pb, ppm	0.0	0.0	0.0	0.0

Experiment 17. Six effluent collections for Experiment 17 were made at the outlet of the pond series. Algae were abundant in most of the samples. Each effluent collection was stored about 6 days and no measurements were made on storage effects.

The test was designed to determine the effect of dissolved oxygen concentration on toxicity of refinery waste waters. Concentrations of effluent used for the test solutions were 100 and 60 percent. Dissolved oxygen was maintained at approximately 3 ppm in one set of solutions and about 7.5 ppm in the other set (Table IX). Differences in oxygen delivery rate did not affect the pH or carbon dioxide content of the test solutions (Table IX).

No deaths occurred in routine tests of 100 percent effluent solutions (Table X) and distress periods ranged from 2 to 6 hours. In the continuous renewal test the distress period lasted for 20 hours in 100 percent concentration and 12 hours in 60 percent. Duration of distress behavior at high and low oxygen levels was similar for comparable effluent concentrations. After the initial distress period, normal behavior prevailed until the fourteenth day when fish in 100 percent concentration became slightly distressed. Food consumption remained normal but by the sixteenth day it was apparent that fish in 100 percent concentration were emaciated. Deaths began in 100 percent, low oxygen solutions, on the eighteenth day and in 100 percent, high oxygen solutions, on the twentieth day (Figure 5). Distress behavior and emaciation became apparent with fish in 60 percent solutions by the twentieth day. Extensive deaths began in 60 percent concentrations on the twenty-fourth day at the low oxygen level and on the twenty-sixth at the high oxygen level. Although distress behavior was not severe

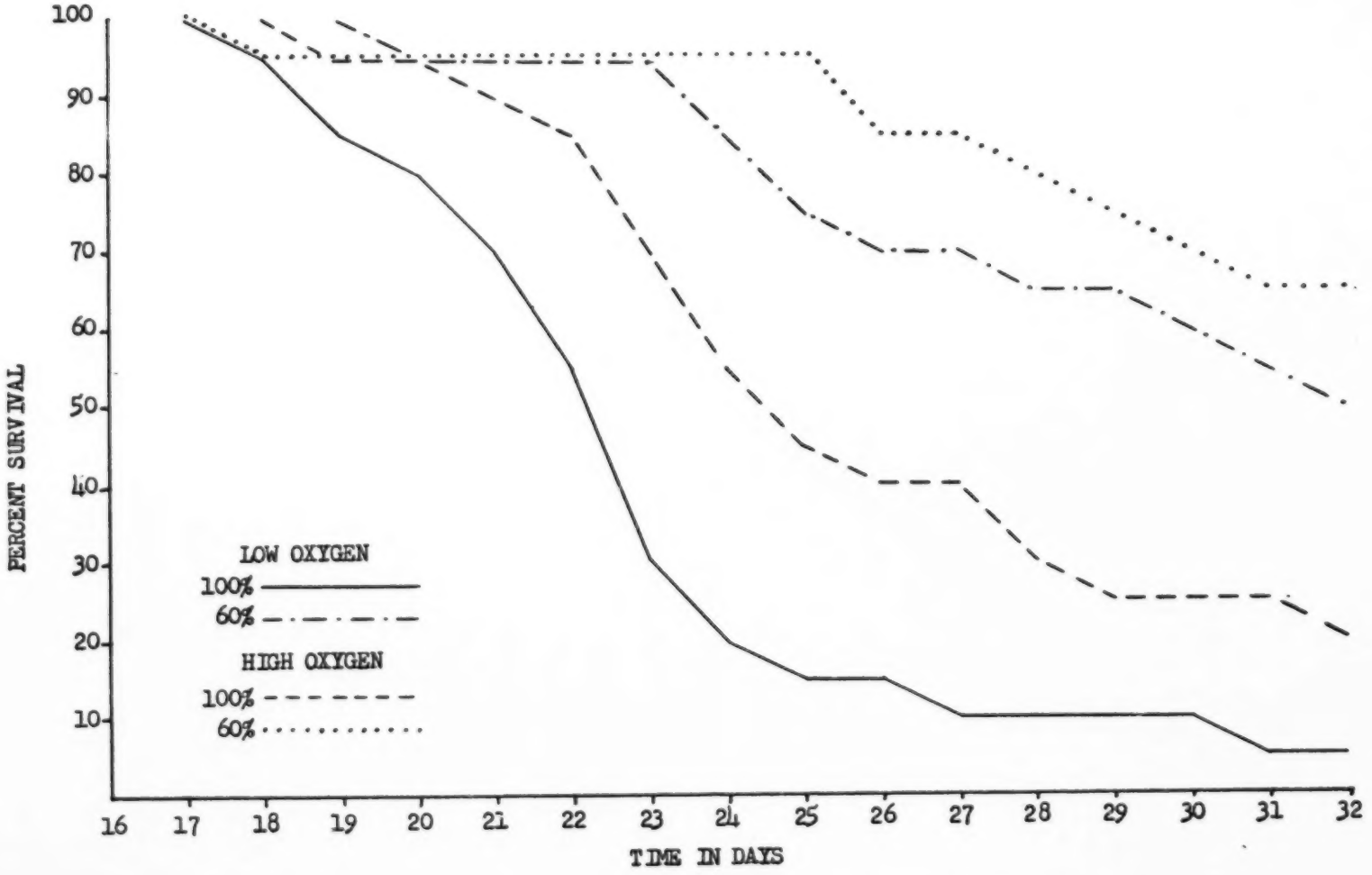

Figure 5. Survival of fish in Experiment 17 with test solutions containing high amounts of dissolved oxygen (7-8 ppm) or low oxygen (3-4 ppm).

and food consumption appeared normal fish gradually became more emaciated. Uniform condition factors (0.52) of dead fish in all effluent solutions show that death occurred when fish reached a certain degree of emaciation.

Increase in dissolved oxygen from about 3 to 7.5 ppm decreased total mortality 15 percent in both concentrations. The 40 percent dilution decreased mortality about 40 percent.

Experiment 18. Effluent for Experiment 18 was obtained when the catalytic crackers of Refinery D were not in operation. The first effluent collection contained 5.5 ppm ammonia indicating that some waste water from the catalytic cracker was present (Table VIII). No ammonia was found in the next three effluent collections. The test was terminated on the twenty-fourth day when waste water from the catalytic crackers appeared in the effluent.

No deaths occurred in routine or long-term tests (Table X). There were no significant differences between mean condition factors of fish in the effluent solutions and the controls. Carbon dioxide concentration ranged from 8 to 17 ppm in 100 percent effluent solution indicating that toxic components other than carbon dioxide contributed to chronic effects observed with effluents of initial low toxicity.

The preceding experiments show that fish were adversely affected by extended exposure to sub-acute concentrations of effluents with initial low toxicity. In ten experiments 96-hour survival was about the same when test solutions were renewed as when not renewed. Survival of fish after 16 days exposure was significantly less than in routine tests in six experiments (8, 9, 10, 11, 12, and 15). Mortality in routine tests of the six experiments ranged from zero to 10 percent,

while mortality after 16 days exposure ranged from 25 to 100 percent. Mortality in four 32-day experiments (12, 13, 15, and 17) was significantly greater than in routine tests. In two experiments (13 and 17) deaths did not occur until after 16 days of exposure. In eight of eleven experiments there was appreciably less survival of fish after prolonged exposure than in routine bioassays. Although no significant mortality occurred during prolonged exposure in three experiments, surviving fish in two (14 and 16) were adversely affected as shown by their condition factors. The only effluent of low toxicity which produced no adverse effects after prolonged exposure was collected when the catalytic crackers of the refinery were not in operation.

Storage effects on effluents with initial low toxicity were difficult to evaluate. In eight experiments 95 to 100 percent survival occurred in initial routine tests of 100 percent effluent, and decreases in toxicity during the storage of these effluents could not be measured by the bioassay technique. Periodic routine tests showed that effluents of three experiments decreased in toxicity. The greatest change occurred in the effluent of Experiment 10 where the 96-hour TL_m increased from 60 to 80 percent during 16 days storage. Chemical analysis of some effluents showed that a decrease of toxic components (except carbon dioxide) occurred during storage. Gould and Dorris (1961) found that effluents of intermediate or low toxicity decreased in toxicity during 30 days storage. Although effluents of initial low toxicity apparently changed more than high-toxicity effluents, the effect was partially diminished since storage time was less. Storage time was 16 days in two experiments of low-toxicity effluents. In nine experiments where more than one collection of effluent was used storage time ranged from 6 to 10 days.

DISCUSSION

The continuous flow system is undoubtedly helpful in maintaining constant concentrations of toxic components in test solutions, although some changes in concentration of toxicants occurred, particularly with effluents of initial low toxicity. Some reduction in toxicity may have occurred in the test containers but changes occurring during storage were more important. For a more accurate evaluation of refinery effluents long-term bioassays should be made at the refinery to avoid changes during storage of effluents.

In spite of the reduction of some toxic components, chronic toxication occurred after prolonged exposure in sub-acute concentrations of effluents with initial low toxicity. The effluent components responsible for the chronic effect are not known. It is generally agreed that with chemically complex wastes it is not often possible to attribute toxicity to single components. Literature dealing with toxicity of specific chemicals is extensive but great variation in toxic levels is reported (Doudoroff and Katz, 1950). Discrepancies arise from variations in species of fish used, test conditions, and time of exposure. Even if the minimum lethal levels of components were known, evaluation would be difficult since mixtures often have effects different from individual components (Tarzwell, 1957). Some of the more important factors that may have influenced or contributed to the chronic effects observed with low-toxicity effluents are

discussed in the following section.

The pH values of the test solutions ranged from 7 to 8.8. Most fish can tolerate a pH range from 5 to 9.5 (Doudoroff and Katz, 1950, and Tarzwell, 1957). The pH may have an indirect effect through its influence on the toxicity of certain materials, particularly weak acids and bases. Ammonia becomes more toxic when pH is raised while cyanides become more toxic when pH is lowered (Doudoroff, 1956).

Dissolved oxygen concentration in test solutions ranged from 1.7 to 10.3 ppm. No deaths were associated with the minimum levels. Low concentrations were limited to the first 3 to 4 days of a test and did not persist for more than a few hours. Whitworth (unpubl.) found that Pimephales promelas survived at oxygen concentrations of less than 1.0 ppm for 22 hours at temperatures from 22-26 C.

The effect of exposure to reduced oxygen concentration for extended periods may be of greater significance. Davison et al. (1959), and Herrmann, Warren, and Doudoroff (1962) found that young salmon were adversely affected when held at reduced oxygen levels. Weight gains were depressed at 4 ppm oxygen while fish surviving in concentrations from 2 to 3 ppm consumed little food and lost weight. The effect occurred in many experiments with low-toxicity effluents. Substances in polluted waters may interfere with the ability of fish to extract or utilize oxygen (Tarzwell, 1957). Substances known to affect fish respiration include carbon dioxide, ammonia, and cyanide, all of which were present in some low-toxicity effluents used in the experiments.

Carbon dioxide accumulated in stored effluents of low toxicity and in the test solutions. The highest concentration observed was 26 ppm. In many analyses the end point (pink color using phenolphthalein

indicator) was difficult to observe because of turbidity caused by algae, clay and other substances. This would tend to give higher results. Positive errors also may have been caused by metal salts, such as those of copper and chromium, and by ammonia, sulfide and nitrite components.

Free carbon dioxide can be detrimental to fish because of its toxicity or interference with respiration. Concentrations between 100 and 200 ppm can be rapidly fatal to freshwater fish in well oxygenated water and at 50 ppm or lower carbon dioxide may be lethal after prolonged exposure or with low oxygen tensions (Doudoroff and Katz, 1950). Concentrations under 30 ppm are not believed to be harmful to most fish in the absence of other adverse factors (Tarzwell, 1957). Black, Fry, and Black (1954) found that with an adequate supply of oxygen (150 mm Hg) carbon dioxide did not limit oxygen utilization by fathead minnows until concentrations of 150 to 250 ppm occurred.

Maximum concentrations of ammonia in test solutions of low-toxicity effluents ranged from 6 to 23 ppm. Studies on the toxicity of ammonia have yielded varying results. The California reports on water quality criteria (CWPCB, 1952 and 1954) list lowest lethal concentrations ranging from 1.0 to 30 ppm. Various workers have reported that the toxicity of ammonia increases as the pH is raised. Acute toxicity is directly related to the undissociated ammonium hydroxide in solution which in turn is a function of pH (CWPCB, 1952). It has not been established that ammonium ions have no influence on chronic toxicity (Doudoroff and Katz, 1950). A decrease in the oxygen content of trout blood occurs when ammonia concentration reaches 0.3 ppm (Brockway, 1950). Survival time of fish in a constant concentration of

undissociated ammonia increases as dissolved oxygen increases (Merkens and Downing, 1957).

Phenol concentrations in test solutions of low-toxicity effluents ranged from zero to 2.3 ppm. In eight experiments maximum concentration was less than 0.5 ppm. Concentrations reported to be damaging or lethal to fish vary from less than 1.0 ppm to over 100 ppm while non-harmful concentrations ranged from 0.1 ppm to less than 17.1 ppm (CWPCB, 1952 and 1954).

The experiments with effluents of initial high toxicity showed that fish were not adversely affected during prolonged exposures to sub-acute concentrations. Casualties or chronic toxication occurred in all long-term experiments with effluents of initial low toxicity when the refinery was in complete operation. Effluents with initial high toxicity had not been treated in oxidation ponds and required dilution of 90 percent or more to obtain sub-acute concentrations. Dilution reduced the amount of all potential toxicants in the effluents. Effluents of initial low toxicity had been subjected to varying degrees of treatment in oxidation ponds. This treatment reduced the amounts of substances causing acute toxicity, such as ammonia, phenol and sulfides (Dorris, Copeland, and Patterson, 1961). The treatment might not reduce the concentration of some components, such as cyanide or metal salts, which could cause chronic or cumulative toxicity. Since little or no dilution was required to obtain sub-acute concentrations with treated effluents, the relative amounts of the trace materials would be large.

Analytical tests on trace materials in effluents used in Experiments 16 and 17 indicate that the concentration of copper (trace to

0.13 ppm as Cu) in the effluent of Refinery D may have been sufficient to have caused chronic toxicity in some experiments. In most natural waters copper sulfate concentrations below 0.025 ppm as Cu evidently are not fatal for most common fish species but concentrations below 1.0 ppm as Cu can be rapidly fatal (Doudoroff and Katz, 1953). Copper concentrations (without regard to the anion involved) reported not toxic to most fish range from 0.25 to 1.0 ppm while concentrations reported toxic range from 0.015 to 3.0 ppm (CWPCB, 1952 and 1954). Copper and other heavy metals are less toxic in hard waters and salts of calcium and magnesium counteract copper toxicity (Doudoroff and Katz, 1953). Some heavy metals are synergistic. Mixed solutions of copper and zinc are much more toxic than the simple additive effect of either metal (Doudoroff and Katz, 1953). Prolonged exposure of the common guppy, Lebistes reticulatus, to lead and zinc caused retarded growth, increased mortality, and delayed sexual maturity in concentrations well below those causing acute toxicity (Crandall and Goodnight, 1962). Lead and zinc may have been present in some effluents used in the experiments.

Concentrations of free cyanide as low as 0.05 ppm have been shown to be toxic to fish held in continually renewed solutions (CWPCB, 1954). Some cyanide complexes (as zinc cyanide) are more toxic than sodium cyanide alone but the toxicity of ionic copper can be reduced by complexing with cyanide (Doudoroff, 1956). Cyanide inhibits the transfer of oxygen from blood to tissues and gills become brighter red than normally (CWPCB, 1952). The gills of distressed and dead fish did not appear different from the gills of control fish. Cyanide is produced in the catalytic cracker and although the maximum concentration found

in effluents of Experiments 16 and 17 was 0.008 ppm it may, at times, be present in sufficient quantity to be toxic.

Lead was not found in the effluents used in Experiments 16 and 17. Lead has been reported as a component of some refinery wastes (Turnbull, DeMann, and Weston, 1954). Concentrations of chromium, arsenic and thiocyanate found in effluents were below those reported toxic to fish.

Many factors must be considered in determining the concentration of an effluent discharge which will be safe for aquatic life (Henderson and Tarzwell, 1957). Included are relative sensitivity of fish species at various life history stages, sensitivity of aquatic organisms important as a food supply, thermal and chemical characteristics of the receiving stream, and duration of exposure. When refinery effluents having 96-hour TL_m values near 10 percent were diluted to sub-acute levels, test fish were not adversely affected during 32 days of exposure. Chronic or cumulative toxication occurred during prolonged exposure with treated refinery effluents having no acute toxicity. Dilution required to prevent chronic toxication in such effluents appeared to be at least 60 percent.

Results of the study show the importance of long-term bioassays in evaluating toxic effects of pollutants. Median tolerance limits of low-toxicity effluents were less in long-term tests than in routine or short-term bioassays. Fish surviving prolonged exposure in low-toxicity effluents were emaciated. If effects on growth or reproduction were considered, or if the exposure period was longer, dilution greater than 60 percent may have been necessary to prevent chronic or cumulative toxication.

SUMMARY

1. The effect on fish of prolonged exposure (16 or 32 days) to effluents from four oil refineries was studied.

2. Effluents from two refineries received little treatment and had relatively high initial toxicity while extensively treated effluents from other refineries were of low initial toxicity.

3. Chemical characteristics of effluents and test solutions are presented.

4. A constant-flow apparatus, capable of renewing 10 liters of test solution every 5.5 hours, was constructed for long-term bioassays.

5. Toxicity of effluents with initial high toxicity did not change appreciably during storage.

6. Low-toxicity effluents decreased in toxicity during storage.

7. Results of long-term bioassays are compared to routine (short-term) bioassays.

8. Fish were not adversely affected by extended exposure in sub-acute concentrations of high-toxicity effluents.

9. Prolonged exposure in sub-acute concentrations of low-toxicity effluents resulted in appreciably less fish survival than in short-term bioassays.

10. Condition factors of fish surviving extended exposure in sub-acute concentrations of low-toxicity effluents were significantly less than control fish.

11. Dilution required to prevent chronic toxication appeared to be at least 60 percent.

12. Effluent components responsible for the chronic toxication are not known but the effect of potential toxicants is discussed.

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